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Dose-dependent changes in neuroinflammatory and arachidonic acid cascade markers with synaptic marker loss in rat lipopolysaccharide infusion model of neuroinflammation

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Abstract

Background: Neuroinflammation, caused by six days of intracerebroventricular infusion of bacterial lipopolysaccharide (LPS), stimulates rat brain arachidonic acid (AA) metabolism. The molecular changes associated with increased AA metabolism are not clear. We examined effects of a six-day infusion of a low-dose (0.5 ng/h) and a high-dose (250 ng/h) of LPS on neuroinflammatory, AA cascade, and pre- and post-synaptic markers in rat brain. We used artificial cerebrospinal fluid-infused brains as controls.

Results: Infusion of low- or high-dose LPS increased brain protein levels of TNF α , and iNOS, without significantly changing GFAP. High-dose LPS infusion upregulated brain protein and mRNA levels of AA cascade markers (cytosolic cPLA₂-IVA, secretory sPLA₂-V, cyclooxygenase-2 and 5-lipoxygenase), and of transcription factor NF- κ B p50 DNA binding activity. Both LPS doses increased cPLA₂ and p38 mitogen-activated protein kinase levels, while reducing protein levels of the pre-synaptic marker, synaptophysin. Post-synaptic markers drebrin and PSD95 protein levels were decreased with high- but not low-dose LPS.

Conclusions: Chronic LPS infusion has differential effects, depending on dose, on inflammatory, AA and synaptic markers in rat brain. Neuroinflammation associated with upregulated brain AA metabolism can lead to synaptic dysfunction.

Keywords: Arachidonic acid, Cytokine, Synapse, Drebrin, Lipopolysaccharide, Synaptophysin, Phospholipase A₂, Neuroinflammation, NF- κ B

Background

Neuroinflammation has been reported in many progressive neurodegenerative and neuropsychiatric brain illnesses, such as Alzheimer's disease, Parkinson's disease, HIV-1 dementia and bipolar disorder [1-4]. Low-grade neuroinflammation can induce slow progressive cellular and tissue damage, whereas high-grade inflammation is associated with robust cytokine release. Despite its pathophysiologic significance, molecular mechanisms underlying effects of low-grade and high-grade neuroinflammation are not fully understood.

Six or 30 days of intracerebroventricular (icv) low-dose bacterial lipopolysaccharide (LPS) (0.5-1.0 ng/h) infusion in rats produces behavioral deficits, induces amyloid deposits, and activates microglia and astrocytes [5,6]. Low-dose LPS infusion in rats increases arachidonic acid (AA, 20:4n-6) turnover in brain phospholipids, brain activities of AA-selective cytosolic Ca²⁺-dependent phospholipase A₂ (cPLA₂) type IVA and of secretory phospholipase A₂ (sPLA₂) type II, and concentrations of prostaglandin (PG) E₂, PGD₂ and thromboxane (TX)B₂ metabolites [6-9]. However, net brain cyclooxygenase (COX) activity, COX-1 and COX-2 protein levels, and calcium-independent phospholipase A₂ (iPLA₂-VI) activity are unchanged with low-dose LPS infusion [6,8]. Depending on the infusion rate and duration, LPS infusion

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has been reported to activate rat brain microglia and astrocytes, increase expression of the transcription factor nuclear factor-kappa B (NF- κ B) and inflammatory cytokines, stimulate microglial inducible nitric oxide synthase activity (iNOS) to produce nitric oxide, and increase brain glutamate [10,11].

A 6-day icv infusion of high-dose LPS (250 ng/h) has been shown to increase activated microglia in the rat thalamus [12], activities of cPLA₂-IVA and sPLA₂-IIA, and unesterified AA and PGE₂ concentrations in rat brain [8]. The same high-dose LPS infusion for 28 days increased mRNA levels of interleukin-1 beta (IL-1 β) and of tumor necrosis factor-alpha (TNF α), reduced pyramidal cells in layers II and III of the entorhinal cortex, attenuated long-term potentiation (LTP), and impaired spatial memory in adult rats [10,13].

Synaptic proteins such as synaptophysin, drebrin and post-synaptic density-95 (PSD-95) play important roles in synaptic plasticity. Drebrin is an actin-binding neuron-specific protein [14], abundant within dendritic spines at postsynaptic excitatory synapses [15]. Suppressing drebrin expression reduces spine density and results in the formation of thin immature dendritic spines [16]. Thus, the drebrin-actin complex plays a crucial role in the regulation of dendritic spine morphology. Synaptophysin is a 38-kD glycoprotein localized in presynaptic vesicle membranes. Functions of synaptophysin include docking, fusion, and endocytosis, otherwise known as membrane trafficking [17]. PSD-95 is a neuronal protein that associates with receptors and cytoskeletal elements at synapses, and is involved in regulating the number and size of dendritic spines and developing glutamatergic synapses [18]. Changes in these synaptic markers have been used to evaluate neuronal damage [19].

The impact of low- and high-dose LPS infusion on brain AA cascade, neuroinflammatory and synaptic markers has not been examined consistently. Therefore, we thought of interest to measure effects of the two doses of LPS on AA, neuroinflammatory and synaptic markers in rat brain after a six-day LPS infusion, compared to infusion of artificial cerebrospinal fluid (aCSF). Based on reported upregulated brain AA metabolism in the LPS-infused rat brain, we hypothesized that 6-day icv infusion of low- and high-dose LPS would increase expression of AA cascade and neuroinflammatory markers, and reduce pre- and post-synaptic markers such as synaptophysin and drebrin, in a dose-dependent manner. An abstract of some of this work has been presented [20].

Methods

Animals

The study was conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 86–23), and was

approved by the Animal Care and Use Committee of the Eunice Kennedy Shriver National Institute of Child Health and Human Development. Three-month-old male Fischer F344 rats (Taconic Farms, Rockville, MD) (n = 24) were housed in a facility with controlled temperature, humidity, and 12-hour light/dark cycle. Food (Rodent NIH-31 auto 18–4 diet, Zeigler Bros, Gardners, PA) and water were provided *ad libitum*. The diet contained (as % of total fatty acid) 20.1% saturated, 22.5% monounsaturated, 47.9% linoleic, 5.1% α -linolenic, 0.02% AA, 2.0% eicosapentaenoic, and 2.3% docosahexaenoic acid [21].

LPS infusion

Low-dose (1 μ g/ml infused at a rate of 0.5 ng/h) or high-dose (0.5 mg/ml infused at a rate of 250 ng/h) of LPS was infused icv in rats for 6 days as described previously [6–9]. The rationale for choosing the 6-day infusion period is based on a pilot study performed when measuring AA incorporation into brain at 2, 3, 4, 6, 8, and 10 days of low-dose LPS infusion. The study found no increase in total AA incorporation over control values until day 4 (10–15% increase) of infusion. Incorporation of AA reached a maximum at day 6 and remained elevated until day 10 [22]. We chose to infuse LPS into the fourth ventricle based on earlier studies that showed activation of microglial cells by such LPS infusion [23] and of unregulated brain AA cascade markers [7–9]. Briefly, the prefilled pump was placed in sterile 0.9% NaCl at 37°C overnight before surgery to start immediate pumping. aCSF (140 mmol/L NaCl, 3.0 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.0 mmol/L MgCl₂, and 1.2 mmol/L NaPO₄, pH 7.4) or LPS from *Escherichia coli* (Sigma, Saint Louis, MO; serotype 055:B5; source strain, CDC 1644–70; chemotype, rough type) at a low or high dose was infused into the fourth ventricle through the cannula via an osmotic pump (Alzet, Model 2002, Cupertino, CA). Postoperative care included triple antibiotic ointment applied to the wound; 5 ml of sterile 0.9% NaCl was injected subcutaneously to prevent dehydration during recovery. Following 6 days of LPS or aCSF infusion, a rat was anesthetized with an overdose of CO₂, and decapitated. The brain was rapidly excised, frozen in 2-methylbutane at –50°C, and stored at –80°C until use. The whole brain was cut into two hemispheres. One half of the brain was used for preparing cytosolic and nuclear extracts and the other half was used for extracting total RNA. Protein homogenates were prepared from the cerebrum and cerebellum without including the brainstem.

Preparation of cytosolic and nuclear fractions

Cytosolic and nuclear extracts were prepared from control (aCSF), low- and high-dose LPS-infused rats, as previously described [24]. Briefly, brains were homogenized in 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 10 mM KCl, and a

protease inhibitor cocktail (Roche, Indianapolis, IN), using a Teflon-glass homogenizer. After adding 0.5% tergitol type NP-40, five additional strokes of homogenization were performed. The suspension was incubated for 30 min on ice, and then centrifuged in a microcentrifuge ($13,000 \times g$, 1 min, 4°C). The resulting supernatant was used as the cytosolic fraction. To the nuclear pellet, solution B (20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.4 M NaCl) and a protease inhibitor cocktail (Roche) were added. The samples were mixed and placed on a small rotatory shaker for 30 min, then centrifuged at $13,000 \times g$ for 3 min at 4°C . The supernatant containing the proteins from the nuclear extracts was transferred to a fresh tube. Protein concentrations of cytosolic fractions and nuclear extracts were determined using Bio-Rad Protein Reagent (Bio-Rad, Hercules, CA).

Western blot analysis

Proteins from the cytosolic and nuclear fractions (65 μg) were separated on 4-20% SDS-polyacrylamide gels (PAGE) (Bio-Rad) and then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). Cytosolic protein blots were incubated overnight in TBS containing 5% nonfat dried milk and 0.1% Tween-20, with specific primary antibodies for IL-1 β (1:500), TNF α (1:500), glial fibrillary protein (GFAP) (1:1000), CD11b (1:1000), ionized calcium-binding adapter molecule 1Iba-1 (1:1000) (monoclonal), iNOS (1:1000), phosphorylated p38 mitogen-activated protein kinase (MAPK) (1:1000) (R&D Systems, Minneapolis, MN), cPLA₂-IVA, sPLA₂-IIA, sPLA₂-V, iPLA₂-VIA, COX-1 (1:1000), COX-2 (1:500), cytochrome P450 epoxygenase (CYP2B1), 5-, 12-, and 15-lipoxygenase (LOX) (1:1000), PSD-95 (1:1000), drebrin (1:1000) (Santa Cruz, Santa Cruz, CA), synaptophysin, (1:1000), and β -actin (1:10,000) (Sigma Aldrich, St. Louis, MO). Nuclear blots were incubated overnight in TBS containing 5% nonfat dried milk and 0.1% Tween-20, with specific primary antibodies for specificity protein 1 (SP-1) (1:500) and nuclear export factor (NXF) (1:500) (Abcam, Cambridge, MA). Cytosolic blots were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Bio-Rad) and were visualized using a chemiluminescence reaction (Amersham, Piscataway, NJ) on X-ray film (XAR-5, Kodak, Rochester, NY). Optical densities of immunoblot bands were measured using Alpha Innotech Software (Alpha Innotech, San Leandro, CA) and were normalized to β -actin (Sigma) to correct for unequal loading. All experiments were carried out with 8 independent samples. Values are expressed as percent of control.

Total RNA isolation and real time RT-PCR

Total RNA was isolated from half-brains using an RNeasy lipid tissue mini kit (Qiagen, Valencia, CA).

Briefly, tissue was homogenized in Qiagen lysis solution and total RNA was isolated by phenol-chloroform extraction. Complementary DNA was prepared from total RNA using a high-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). mRNA levels (cPLA₂-IVA, sPLA₂-IIA and -V, iPLA₂-VIA, COX-1, COX-2, 5-, 12-, 15-LOX, cytochrome P450 epoxygenase, drebrin, synaptophysin) were measured by quantitative RT-PCR, using an ABI PRISM 7000 sequence detection system (Applied Biosystems). Specific primers and probes for these markers, purchased from TaqMan[®] gene expression assays (Applied Biosystems), consisting of a 20X mix of unlabeled PCR primers and Taqman minor groove binder (MGB) probe (FAM dye-labeled). The fold-change in gene expression was determined by the $\Delta\Delta C_T$ method [25]. Data are expressed as the relative level of the target gene in the LPS-infused rat normalized to the endogenous control (β -globulin) and relative to the control (calibrator). All experiments were carried out in duplicate with 8 control and 8 brain samples from LPS-infused rats and data are expressed as relative expression.

Transcription factor NF- κ B p50 and NF- κ B p65 activity

Nuclear extracts were assayed for brain NF- κ Bp50 and NF- κ Bp65 activities according to the manufacturer's instructions (Panomics, Fremont, CA). Briefly, 10 μg of nuclear extract was preincubated with biotin-labeled NF- κ B p50/p65 for 60 min in a microfuge tube. The labeled oligonucleotide-nuclear protein complexes were transferred to immobilized streptavidin-coated 96-well plates. The bound oligonucleotide protein complex was detected by using a specific primary antibody directed against either NF- κ B p50 or p65, followed by addition of HRP-conjugated secondary antibody. Color was developed by adding tetramethylbenzidine substrate, and optical densities were measured at 450 nm. Values ($n = 8$) are expressed as percent of control.

Statistics

Data are presented as mean \pm SEM. When three groups were compared (low-dose LPS, high-dose LPS and aCSF control), statistical significance was determined using a one-way ANOVA with Newman-Keuls Multiple Comparison post-hoc test for multiple comparisons between the groups. Statistical significance was set at $p \leq 0.05$.

Results

Body weight

Body weight (grams) was significantly reduced by 9% and 19% in low- (264 ± 7 ; $p < 0.05$) and high- (235 ± 5 ; $p < 0.001$) dose LPS-infused rats, respectively, compared to control (290 ± 7), as previously reported [8].

Brain microglia, astrocyte markers and proinflammatory cytokines

Six days of high-dose but not low-dose LPS infusion significantly elevated the brain protein level (89%) of the microglia/macrophage marker CD11b [26], compared to control (Figure 1A). Consistent with that, a significant increase in the protein level of microglia marker Iba-1 was found in high-dose but not low-dose LPS infused rats (Figure 1B). The astroglial marker GFAP protein level was unchanged in rats infused with either LPS dose compared to control (Figure 1C). Proinflammatory cytokine TNF α protein levels were significantly increased independently of the dose of LPS (332% and 340% with low- and high-dose LPS, respectively) (Figure 1D), whereas the IL-1 β protein level was unaltered (Figure 1E). The protein level of iNOS, a major free radical-generating enzyme in activated microglia [27], was increased significantly by 180% and 155%, with the low- and high-LPS doses, respectively (Figure 1F). These significant protein changes in CD11b, TNF α and iNOS did not correspond to significant changes in their respective mRNA levels (data not shown). Further, there was no significant difference between low and high-dose LPS infused rats in any neuroinflammatory marker.

Brain arachidonic acid cascade markers

High-dose LPS significantly increased phosphorylated cPLA₂-IVA (120%) as did low-dose LPS (60%) ($p < 0.05$) compared to control (Figure 2A). The protein and mRNA

levels of cPLA₂-IVA were significantly elevated by 178% and 2-fold, respectively, in rats infused with high-dose LPS (Figures 2B and 2C). High-dose LPS significantly increased phospho-cPLA₂ and cPLA₂ levels compared to low-dose LPS (Figures 2A and 2B). sPLA₂-V protein levels were significantly increased by 34% and 24% in low- and high-dose LPS-infused rats respectively, compared to control (Figure 2D), but without a significant change in sPLA₂-V mRNA (Figure 2E). sPLA₂-IIA and iPLA₂-VIA protein levels were unaltered.

COX-2 protein (61%) and mRNA (5-fold) levels were significantly elevated in rats infused with high- but not low-dose LPS compared to controls (Figures 3A and 3B), whereas COX-1 protein was unchanged compared to control (Figure 3C). High-dose LPS significantly increased both COX-2 protein and mRNA levels compared to low-dose LPS infused rats (Figures 3A and 3B). Protein (74%) and mRNA levels of 5-LOX (8-fold) were significantly increased only with the high-dose LPS infusion (Figures 3C and 3D). Protein levels of 12-LOX, 15-LOX and cytochrome P450 epoxygenase were unaltered by either LPS infusion (Figures 3F and 4A-B).

NF- κ B activity and phosphorylated p38 MAPK

DNA-binding activity of NF- κ Bp50 was significantly increased (40%) in the high-dose LPS-infusion compared to control rats ($p < 0.001$) (Figure 4C), whereas NF- κ Bp65 DNA-binding activity was unaltered (Figure 4D). High-dose LPS significantly increased NF- κ Bp50 activity

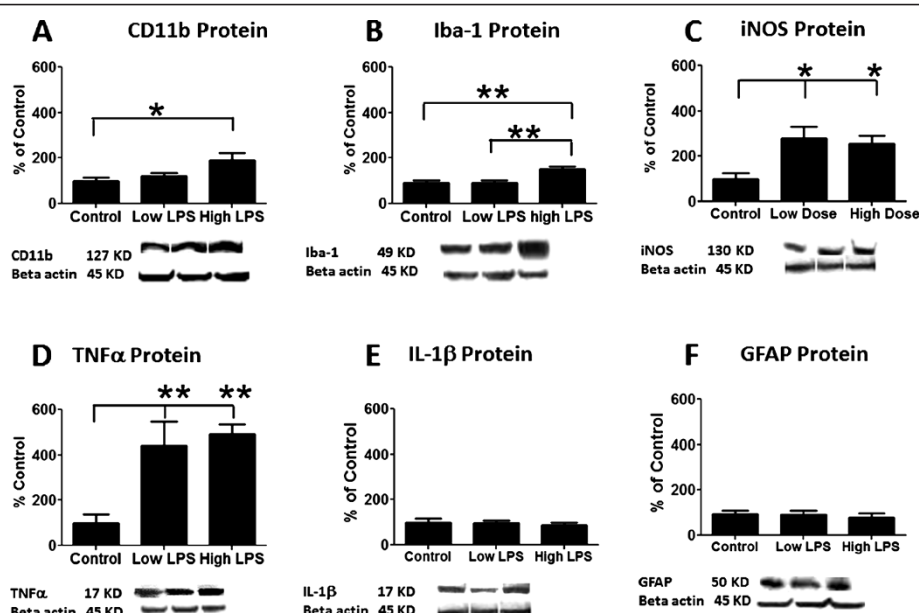


Figure 1 Protein levels of (A) CD11b, (B) Iba-1 (C) iNOS, (D) TNF α , (E) IL-1 β and (F) GFAP (with representative immunoblots) in control, low- and high-dose LPS rat brain. The protein level was determined in a homogenate of cerebrum and cerebellum, as in all subsequent figures (see methods). Data are ratios of optical densities of protein to β -actin, expressed as percent of control. Comparisons between LPS groups were made using one-way ANOVA, mean \pm SEM ($n = 8$). * $p < 0.05$.

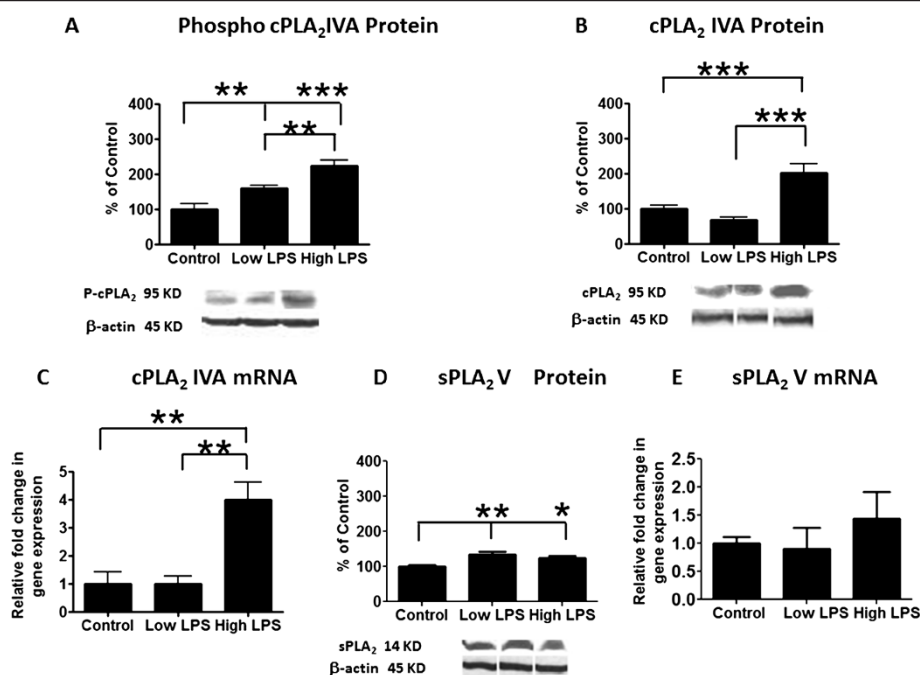


Figure 2 Mean (A) phosphorylated cPLA₂-IVA, (B) cPLA₂-IVA and (D) sPLA₂-V protein levels (with representative immunoblots) in control, low- and high-dose LPS rat brain. Data are ratios of optical densities of protein to β-actin, expressed as percent of control and were compared using one-way ANOVA, mean ± SEM (n=8). mRNA levels of (C) cPLA₂-IVA and (E) sPLA₂-V in control, low- and high-dose LPS in rat brain, measured using real time RT-PCR. Data are normalized to the endogenous control (β-globulin) and relative to control level (calibrator), using the $\Delta\Delta C_T$ method. Comparisons between LPS groups were made using one-way ANOVA, mean ± SEM (n=8), *p < 0.05, **p < 0.01.

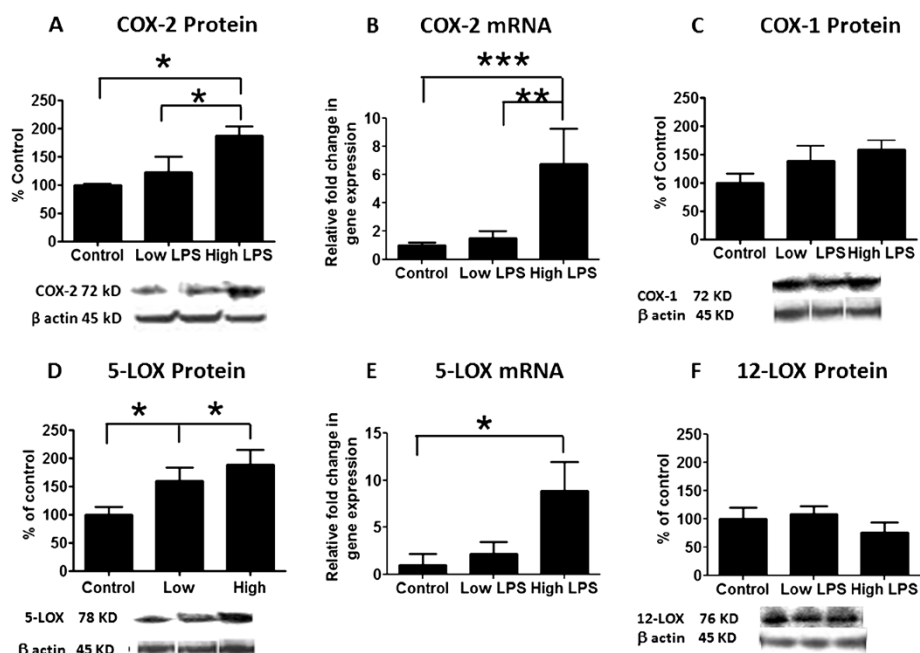


Figure 3 Protein levels of (A) COX-2, (C) COX-1, (D) 5-LOX and (F) 12-LOX (with representative immunoblots) in control, low- and high-dose LPS rat brain. Data are ratios of optical densities of protein to β-actin, expressed as percent of control, and were compared using one-way ANOVA, mean ± SEM (n=8). mRNA levels of (B) COX-2 and (E) 5-LOX in control, low- and high-dose LPS, measured using real time RT-PCR. Data normalized to the endogenous control (β-globulin) and relative to control level (calibrator), using the $\Delta\Delta C_T$ method. Comparisons between LPS were made using one-way ANOVA, mean ± SEM (n=8), *p < 0.05, **p < 0.01.

compared to low-dose LPS infused rats (Figures 2A and 2B). Active phosphorylated p38 MAPK was significantly increased by 108% and 109% with low- and high-dose LPS infusion, respectively, compared to control (Figure 4E).

Synaptic markers

Protein levels of the presynaptic marker synaptophysin were significantly decreased by high-dose (−35%) and low-dose (−16%) LPS, compared to control ($p < 0.05$) (Figure 5A). However, the mRNA level of synaptophysin was significantly reduced (0.7-fold) only by high-dose LPS (Figure 5B). Postsynaptic marker drebrin protein (−30%) and mRNA (−0.6-fold) levels were significantly reduced in high- but not low-dose LPS infused rats compared to control (Figures 5C and 5D). High-dose LPS significantly decreased protein and mRNA levels of synaptophysin and drebrin compared to low-dose LPS infused rats (Figures 5A and D). Further, another marker of post-synaptic marker PSD-95 was significantly decreased by high-dose LPS but not low-dose LPS (Figure 5E). The protein levels of NXF, a transcription factor of drebrin [28], and of SP-1, a constitutive transcription factor of synaptophysin [29], were unaltered (Figures 5F and G).

Discussion

The present study demonstrates that a 6-day icv infusion of low- or high-dose LPS in unanesthetized rats,

compared with aCSF infusion, significantly increased brain levels of AA cascade and neuroinflammatory markers in a dose-dependent manner (Table 1). These changes were associated with decreased levels of pre- and post-synaptic markers in a dose dependent manner as well. Similar changes have been demonstrated in post-mortem brain tissue from patients with bipolar disorder, schizophrenia, AIDS and Alzheimer's disease [4,30-35], suggesting that LPS infusion at different rates in rats are reasonable models for understanding interactions of brain AA metabolism, neuroinflammation and synaptic integrity in these progressive human brain diseases, and perhaps for designing treatments for them [8,9,36,37].

Previously, we reported that a 6-day icv infusion of either LPS dose increased cPLA₂-IV activity, but that the low-dose infusion did not alter the cPLA₂-IV protein level [6-8]. In the present study, although both LPS doses increased phosphorylated cPLA₂-IVA and phosphorylated p38 MAPK (linked to activation and phosphorylation of cPLA₂ and AA release [38]), only the high-dose induced mRNA and protein increases of cPLA₂-IVA. These new data demonstrate that high-dose but not low-dose LPS infusion induced transcriptional level activation. Both doses also increased brain sPLA₂ activity [6-8], which can be ascribed to upregulated protein levels of sPLA₂-V [39,40] since sPLA₂-IIA protein was unchanged. In contrast, Ca²⁺-independent docosa-hexaenoic acid (DHA)-selective iPLA₂-VIA protein and

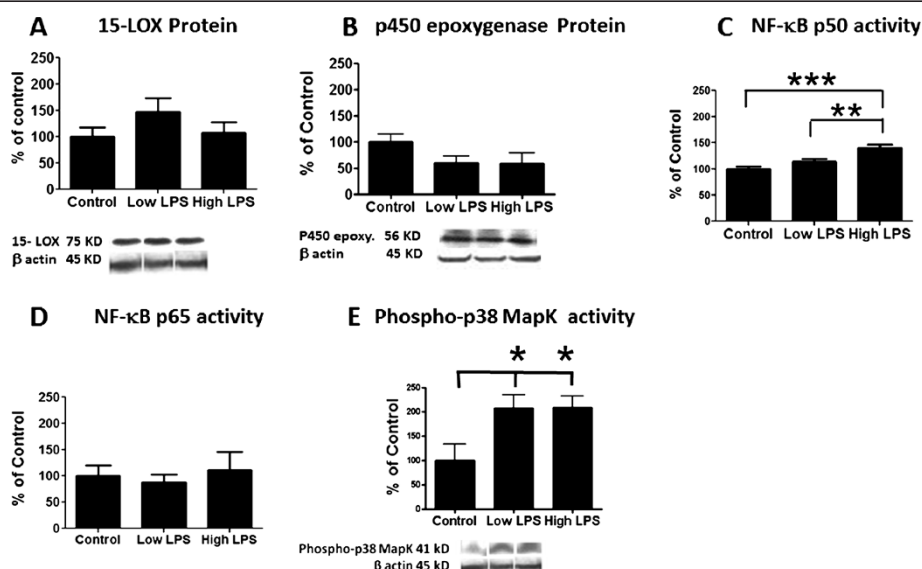


Figure 4 Protein levels of (A) 15-LOX, and (B) p450 epoxygenase (with representative immunoblots) in control, low- and high-dose LPS rat brain. Data are ratios of optical densities of protein to β -actin, expressed as percent of control and were compared using one-way ANOVA, mean \pm SEM ($n = 8$). Representative brain transcription factor binding activities (DNA-protein complex) of (C) NF- κ B p50 and (D) NF- κ B p65 in control, low- and high-dose LPS rat brains. DNA binding activity was measured in brain nuclear extracts as described in Materials and Methods. Mean \pm SEM ($n = 8$), ** $p < 0.01$. Protein levels of (E) phospho-p38 MAPK (with representative immunoblots) in control, low- and high-dose LPS rat brain. Data are ratios of optical densities of protein to β -actin, expressed as percent of control. Comparisons between LPS groups were made using one-way ANOVA, mean \pm SEM ($n = 8$), * $p < 0.05$.

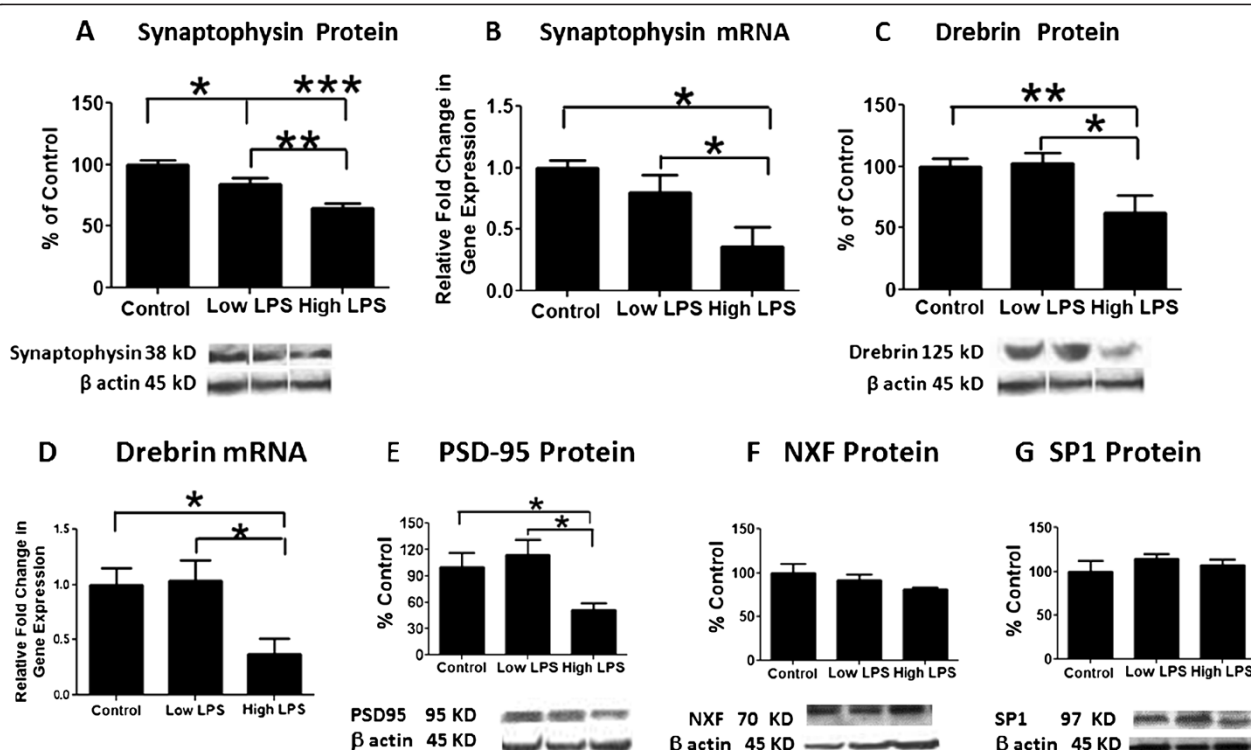


Figure 5 Protein levels of (A) synaptophysin, (C) drebrin and (E) PSD-95 (with representative immunoblots) in control, low- and high-dose LPS rat brain. Data are ratios of optical densities of protein to β -actin, expressed as percent of control and were compared using one-way ANOVA, mean \pm SEM (n = 8). mRNA levels of (B) synaptophysin and (D) drebrin in control, low- and high-dose LPS in rat brain, measured using real time RT-PCR. Data are normalized to the endogenous control (β -globulin) and relative to control level (calibrator), using the $\Delta\Delta C_T$ method. Comparisons between LPS groups were made using one-way ANOVA, mean \pm SEM (n = 8), *p < 0.05, ***p < 0.001.

mRNA levels were not significantly altered by either dose of LPS. This is consistent with evidence that neither LPS infusion dose changed the brain unesterified DHA concentration, DHA turnover in brain phospholipids or iPLA₂-VI activity [6,8,22,41].

The brain PGE₂ concentration was elevated in rats infused with both LPS doses, consistent with increased concentration of unesterified AA released by cPLA₂-IVA and sPLA₂-V [6-8]. Protein and mRNA levels of COX-2, the rate-limiting enzyme in PGE₂ biosynthesis, were up-regulated with only the higher dose of LPS, showing a dose-dependent effect. In line with a previous observation, low-dose LPS infusion did not alter the brain COX-2 protein level [6].

LOX and cytochrome P450 epoxygenase convert AA to 5-hydroxyicosatetraenoic acid (5-HETE)/leukotrienes/lipoxins and epoxyeicosatrienoic acids, respectively. The high-dose LPS infusion increased 5-LOX protein and mRNA levels, without altering 12- or 15-LOX levels. Concentrations of 12-HETE and 15-HETE remain unchanged with both doses [8]. In this regard, LPS has been reported to induce expression of 5-LOX [42] and the 5-LOX-activating protein (FLAP) via NF- κ B-mediated transcriptional mechanisms in mononuclear phagocytes, which is

critical for leukotriene synthesis [43]. Another AA cascade marker, cytochrome P450 epoxygenase, remained unchanged after LPS infusion.

Cell culture studies have shown that two major cytokines, IL-1 β and TNF α , can induce transcription of cPLA₂, sPLA₂, COX-2, and iNOS genes through an NF- κ B-mediated mechanism [44-46], as NF- κ B binding sites are present on the promoter regions of these genes [46-49]. Our study suggesting that increases of cPLA₂-IVA, COX-2, and iNOS in high-dose LPS-infused rats were due to elevated levels of TNF α following increased NF- κ B p50 activity, was recently supported by *in vitro* studies [50]. The response was not observed in low-dose LPS infusion. NF- κ B p50 also is known to regulate transcription of many proinflammatory genes [51,52]. Since p38 MAPK can activate NF- κ B mediated cell signaling [53], the increases found in phosphorylated p38 MAPK (the active form) [54] may be involved in LPS-mediated NF- κ B activation. These changes may induce TNF α and COX-2 [55,56]. NF- κ B also can be activated by other cellular signal transduction factors, such as extracellular signal-regulated kinase (ERK) or c-Jun N-terminal kinase (JNK) [57,58].

Consistent with the increased TNF α level, a pro-inflammatory cytokine produced by microglia [59], high-

Table 1 Summary of effects compared to control, 6 days of LPS infusion on neuroinflammatory, AA cascade, and synaptic markers in rat brain

	Low-dose LPS	High-dose LPS
Neuroinflammation		
TNF α (protein)	↑	↑
CD11b (protein)	No change	↑
Iba-1 (Protein)	No change	↑
iNOS (protein)	↑	↑
AA cascade		
p-cPLA ₂ -IVA (protein)	↑	↑
cPLA ₂ -IVA (protein, mRNA)	No change	↑
sPLA ₂ -V (protein)	↑	↑
COX-2 (protein, mRNA)	No change	↑
5-LOX (protein, mRNA)	No change	↑
Transcription regulators		
NF- κ Bp50 (activity)	No change	↑
p38 MAPK (protein)	↑	↑
Synaptic markers		
Synaptophysin (protein)	↓	↓ (+ mRNA)
Drebrin (protein, mRNA)	↓	↓ (+ mRNA)
PSD-95 (protein)	No Change	↓
IL-1 β , GFAP, iPLA ₂ -VI, sPLA ₂ -IIA, COX-1, 12- and 15-LOX, cytochrome P450 epoxygenase, mPGES, NF- κ Bp65, NFX, and SP-1 were unchanged.		

dose but not low-dose LPS increased the microglia/macrophage marker CD11b, without altering the astroglial marker GFAP. Microglia but not astrocytes express the LPS CD14/toll-like receptor 4 (TLR4) [60], but we cannot rule out localized regional changes in brain GFAP. Indeed, we reported that low-dose LPS increased lectin-reactive microglia in the cerebral ventricular surround, pia mater, and glial membrane of the cortex, and produced morphological changes of GFAP-positive astrocytes in the cortical mantle and areas surrounding the cerebral ventricles [6]. An increase of CD11b with NF- κ B-induced activation of COX-2 by high-dose LPS infusion is consistent with the observation that CD11b plays a significant role in the optimal production of COX-2 via NF- κ B [56]. Since CD11b integrins originally were identified as LPS receptors [61], our data suggest that high-dose but not low-dose LPS modifies the CD11b receptor. Further, the microglial specific marker [62], Iba-1 was significantly increased in high-dose compared to low-dose LPS infused rats, suggesting the presence of activated microglia in high-dose LPS infused rats.

Increases in TNF α and iNOS levels and in AA cascade enzyme expression have been implicated in neuronal damage [63] and cognitive-behavioral impairments in rats infused with high doses of LPS [10]. The latter impairments might be due to reduced expression of the postsynaptic dendritic spine actin-regulatory protein

drebrin, which is involved in spinogenesis and synaptogenesis [15]. LPS reduced the presynaptic vesicle marker synaptophysin in a dose-dependent manner. The molecular mechanisms by which neuroinflammation down-regulate drebrin in rat brain are not clear. Several studies reported that inhibition of p38 MAPK activity prevented cytokine-induced loss of synaptophysin in rat primary cortical neuronal cultures and in an animal model of Alzheimer's disease [64,65]. Similarly, drebrin loss was attributed to p38 MAPK activity in hippocampal cultures [66]. Apart from cytokines, PGE₂ is known to activate p38 MAPK in rat primary astrocytes [67]. Both LPS doses increase the PGE₂ concentration in rat brain [6-8] and may be involved in activating p38 MAPK. Increased p38 MAPK activity observed in this study may explain the loss of synaptophysin following low- and high-dose LPS infusion. Interestingly, the increased p38 MAPK may not be solely responsible for synaptic loss, because low-dose LPS increased p38 MAPK activity without changing the synaptic proteins. This suggests that other mechanisms are involved regulating the synaptic proteins. Further specific *in vitro* studies are required to understand synaptic regulation by LPS. Both LPS doses also did not change protein levels of NXF or SP-1, transcription factors for drebrin and synaptophysin, respectively [28,29]. The changes in the synaptic markers might be related to post-transcriptional regulation or changes of other transcription factors/kinases [28,29]. Consistent with loss of post-synaptic drebrin, the protein level of another postsynaptic marker PSD-95 was decreased in high-dose LPS infused rats compared to controls and low-dose infused rats.

A limitation of current study is that one time point (6 days) was taken to study the effects LPS infusion based on the AA cascade metabolism. This choice was based on a prior study showing that AA incorporation in rat brain with low-dose LPS infusion did not increase until day 4 (10–15% increase) of infusion, reached a maximum at day 6 and remained elevated until day 10; no difference was evident at day 28 [22]. However, at earlier time points Toll receptors and other pathways are activated [68]. Future studies should aim at earlier and perhaps later time points.

Conclusions

We demonstrated that 6-day icv LPS infusion dose-dependently increased neuroinflammatory and AA cascade markers associated with an increase in phosphorylated p38 MAPK, and decreased synaptic markers (Table 1). Low-dose LPS increased TNF α , iNOS, sPLA₂-V, cPLA₂-IVA and p38 MAPK phosphorylation, and reduced presynaptic synaptophysin. High-dose LPS upregulated gene expression of AA cascade enzymes via NF- κ B, CD11b, and down-regulated postsynaptic drebrin. Targeting these disturbed

pathways by specific anti-inflammatory drugs and/or cPLA₂ inhibitors could lead to therapeutic treatments of neuronal damage and behavioral changes associated with neuroinflammation [7-9,69].

Abbreviations

aCSF, Artificial CSF; cPLA₂, Calcium-dependent cytosolic phospholipase A₂; COX, Cyclooxygenase; GFAP, Glial fibrillary acidic protein; HETE, Hydroxyeicosatetraenoic acid; IL-1β, Interleukin-1β; iNOS, Inducible nitric oxide synthase; iPLA₂, Calcium-independent phospholipase A₂; LOX, Lipoxygenase; LPS, Lipopolysaccharide; MAPK, Mitogen-activated protein kinase; NF-κB, Nuclear factor-kappa B; NXF, Nuclear export factor; p, Phosphorylated; mPGES, Membrane PGE synthase; sPLA₂, Secretory phospholipase A₂; SP-1, Specificity protein 1; TNFα, Tumor necrosis factor α.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MK participated in the sacrifice of animals, carried out the molecular biology studies, performed the statistical analysis, and drafted the manuscript. MB wrote the animal protocol, participated in the rat surgery, prepared osmotic pumps, and revised the manuscript. VLK performed Western blot studies. MC performed the surgery. SIR participated in the design of the experimental work and corrected the final version of the manuscript. JSR participated in the design of the experimental work, performed the statistical analysis, drafted the manuscript and wrote its final version. All authors read and approved the final manuscript.

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